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A modified HPLC method to detect salicylic acid in must and wine after its application in the field to induce fungus resistance

by

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S u m m a r y : Since the application of salicylic acid (SA) to induce systemic acquired resistance (SAR) in plants is currently discussed as an alternative for copper against downy mildew (*Plasmopara viticola*), a sensitive HPLC method with UV/Vis-DAD-detection was developed to determinate SA in must and wine. The rate of recovery was 92 % at a level of 0.15 mg·l^{-1} with a detection limit of 0.003 mg·l^{-1}. We have analyzed several musts and wines from field experiments with SA application and have compared their SA concentrations with 23 commercially available German wines. Nearly all samples contained small amounts of SA. The mean concentration in white and red wines was 0.05 mg·l^{-1} (0.11 mg·l^{-1} max.) and 0.16 mg·l^{-1} (0.43 mg·l^{-1} max.), respectively. Application of SA downy mildew control did not increase the amounts of SA in must or wine.

K e y w o r d s : salicylic acid, HPLC, wine, must, systemic acquired resistance (SAR).

Introduction

Salicylic acid (SA) is known to be involved in plant defense mechanisms against fungal diseases as a hormone-like messenger (Raskin 1992; Ryals *et al.* 1996) and has been implicated as one of the polyphenolic constituents of wines exerting beneficial effects on human health (Muller and Fugelsang 1994). Literature on its concentration in grape and wine is rather contradictory, values range from < 0.02 to 21.5 mg·l^{-1} probably due to methodical differences. As early as 1903, Traphagen and Burke reported 0.32 mg·kg^{-1} SA in fresh Concord grapes, Guimberteau and Portal (1961) and Ribereau-Gayon (1963) found maximal 1.5 mg·l^{-1} SA in wines using paper chromatography. In more recent studies, methods based on gas or liquid chromatography have been published (Robertson 1983; Bianco and Marucchi 1990; Shi and Schwedt 1995; Janssen *et al.* 1996). Despite the analytical advances, SA values reported for grapes and wines vary over a wide range and can differ nearly 100-fold (Tab. 1). Haeberle (1987) referring to the values reported by Swain *et al.* (1985), claimed that the higher specificity of modern methods like HPLC may ultimately result in smaller SA values. On the other hand, Janssen *et al.* (1998) concluded that coelution during the HPLC analysis might have been responsible for these large differences. Venema *et al.* (1996), using fluorescence detection which is more sensitive and specific than UV/Vis-detection to determine SA in grape samples, detected only negligible amounts. The highest SA concentrations were found by Muller and Fugelsang (1994) but they did not outline the analysis procedure. Testing some of the published HPLC methods indicated problems with coelution; thus we developed a new HPLC method to achieve higher resolution and to avoid coelution. This method was used to study the SA content in commercially available wines.

T a b l e 1

Salicylic acid concentration of berries and wines as determined by different analytical methods

Product	Salicylic acid	References
Berries	9.4 mg·kg^{-1}	Haeberle 1987
Berries	0.04 mg·kg^{-1}	Robertson 1981
Berries	0.04-0.08 mg·kg^{-1}	Robertson 1983
Berries	1.6-18.6 mg·kg^{-1}	Swain *et al.* 1985
Berries	< 0.02 mg·kg^{-1}	Venema *et al.* 1996
Wine	0.1-1.0 mg·l^{-1}	Drawert *et al.* 1976
Wine	11.0-21.5 mg·l^{-1}	Muller *et al.* 1994
Wine	0.26-0.68 mg·l^{-1}	Venema *et al.* 1996

Another objective was to quantify SA in must and wine from field experiments where SA had been used to induce systemic acquired resistance (SAR) against downy mildew as an alternative for the application of copper (Wöhrle 1997; Kornitzer 1998). SA has previously been shown to act as an elicitor of phenolics and hydrolytic enzymes involved in defense reactions of grapevines against fungus attack (Renault *et al.* 1996; Busam *et al.* 1997; Kraeva *et al.* 1997); however it is still unknown whether or not exogenously applied SA will accumulate in must and/or wine. Despite the assumed beneficial health effects of SA reported by Muller and Fugelsang (1994), high rates of SA uptake in food may increase the risk of hypersensitivity (Haeberle 1987).

Material and Methods

M u s t s a n d w i n e s : As commercial samples 23 white wines from 6 grape-growing regions in Germany

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were selected. In addition, 10 must and 6 wine samples originating from grapes of experiments performed in 1997 and 1998 with different phytosanitary treatments were analyzed ("experimental sample").

Grapes were grown in the Rheingau and Baden regions (Germany) to compare three systems of plant protection management: (a) "organic" (according to the EC 2092/91 regulation of Organic Agriculture in the European Community), (b) "organic with the substitution of copper by SA", and (c) "integrated" (using chemical fungicides) (WÖHRLE 1997, KORNITZER 1998). SA (99.82 %, Merck, Darmstadt, Germany) was applied in aqueous solution and was tested in 1997 on the cvs Riesling and Müller-Thurgau and in 1998 on Riesling and Pinot noir. Total amounts of SA applied in 1997 were 2.76 kg·ha^{-1} for Riesling (8 treatments of 0.18-0.36 kg·ha^{-1} each) and 4.2 kg·ha^{-1} for Müller-Thurgau (13 treatments of 0.18-0.48 kg·ha^{-1} each). In 1998, 2.86 kg·ha^{-1} were used for Riesling (8 treatments of 0.18-0.48 kg·ha^{-1} each) and 4.2 kg·ha^{-1} for Pinot noir (12 treatments of 0.18-0.48 kg·ha^{-1} each). In addition, wine samples originating from varietal trials with Cabernet Sauvignon, Dornfelder, Dunkelfelder, Pinot noir, and Pinot noir précoce were analyzed at the Research Institute at Geisenheim. Must samples were stored at -20 °C until analysis. Wine samples were stored at +13 °C.

Chemicals: Acetonitrile (gradient grade), hydrochloric acid, Extrelut 20®, polypropylene columns (50 ml) incl. filters and drain tubes, phosphoric acid, salicylic acid, and sodium chloride (Merck, Darmstadt, Germany). Chloroform, methanol, and potassium dihydrogenphosphate (Fluka, Buchs, Switzerland).

Sample preparation: Must and wine samples were prepared using a 50 ml polypropylene column equipped with filters and a drain tube. The column was filled with Extrelut 20® to remove sugars, amino and organic acids, and proteins which can interfere with the SA analysis when using a UV/VIS-detector at a wavelength of 235 nm (Fig. 3). For red wine or must, the Extrelut was first mixed with 5 g of sodium chloride. The Extrelut material was then rinsed with 50 ml of acetonitrile pre-cleaning and a sub-sample of 20 ml of wine or must was adjusted to pH 2.0 with conc. hydrochloric acid and given onto the dry Extrelut column. The volume did not exceed 22 ml; 15 min after adding the wine sample, SA was eluted with 50 ml of chloroform/methanol (90:10, v:v). This solution was concentrated to dryness, the residue dissolved in 1 ml mobile phase (20-fold concentrated), and injected into the HPLC column.

HPLC analysis: A Merck L-6200 Intelligent Pump was connected to a Shimadzu SPD-10AV*vp* UV/Vis-Detector (wavelength: 235 nm), and a Waters 991 Photodiode Array Detector (190-600 nm Scan, 235 nm Baseline). A Merck LiChroCart® Superspher® 60 RP-select B column, 250 x 4 mm, 5 µm particle size, was maintained at 50 °C with a Biorad Column Oven. Isocratic elution was performed with 0.025 M phosphoric buffer adjusted to pH 2.0 with phosphoric acid/acetonitrile (85:15, v:v) with a flow of 1.0 ml·min^{-1}. A Merck AS-2000A autosampler was used for the injection of 10 µl samples. All wine samples were extracted and injected twice.

Results and Discussion

Characterization of the method: The modified HPLC method was calibrated by external standardization. Solutions of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 mg·l^{-1} of SA were prepared and injected 4 times. The resulting calibration curve is shown in Fig. 1. The correlation coefficient between a known amount (AMT) of SA and the resulting peak area at 235 nm was 0.99. The limits of detection and quantification were determined according to Hewlett Packard (ANONYMOUS 1993). The limit of detection was 0.003 mg·l^{-1} (threefold baseline noise), the limit of quantification was 0.01 mg·l^{-1} (10-fold baseline noise). In order to test the rate of recovery, we added SA solutions of 0.07 and 0.15 mg·l^{-1}, respectively, to a wine without a measurable SA concentration. Each solution was prepared 4 times and replicated injections were performed on each sample. The recovery was 92 % at a level of 0.15 mg·l^{-1} (standard deviation 0.01 mg·l^{-1}, relative variation coefficient 7.5 %). Fig. 2 shows a typical HPLC chromatogram of a white wine spiked with 0.15 mg·l^{-1} SA.

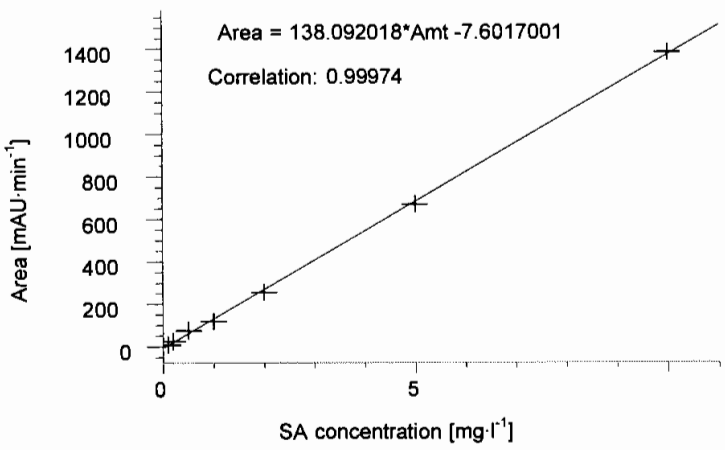

Fig. 1: Calibration curve to determine salicylic acid (SA). Peak area [mAU·min^{-1}] at 235 nm and the amount (Amt) of SA added for calibration.

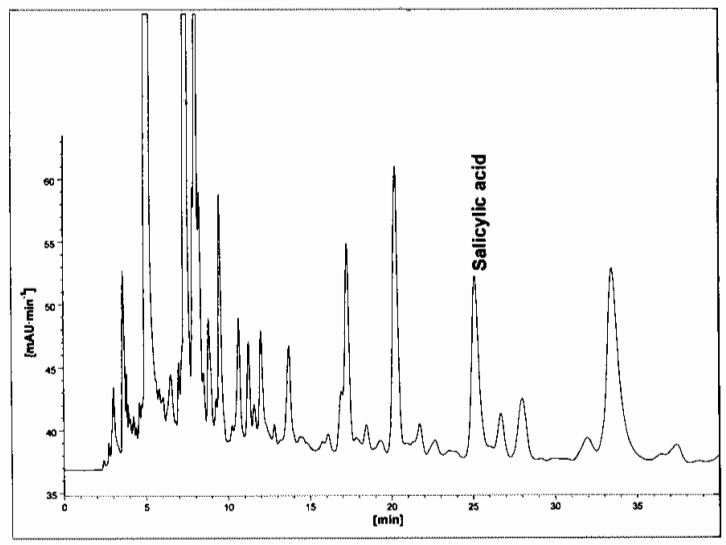

Fig. 2: HPLC/UV chromatogram (235 nm) of a white wine after addition of 0.15 mg·l^{-1} salicylic acid.

The SA peak was well separated from the other peaks and no coelution was observed. In comparison to the method of VENEMA *et al.* (1996), the use of Extrelut 20® lead to

purged extracts. The diode array detector was used to verify the presence of SA and to prove the peak purity. The UV/Vis-spectrum of SA dissolved in the mobile phase at pH 2 is shown in Fig. 3.

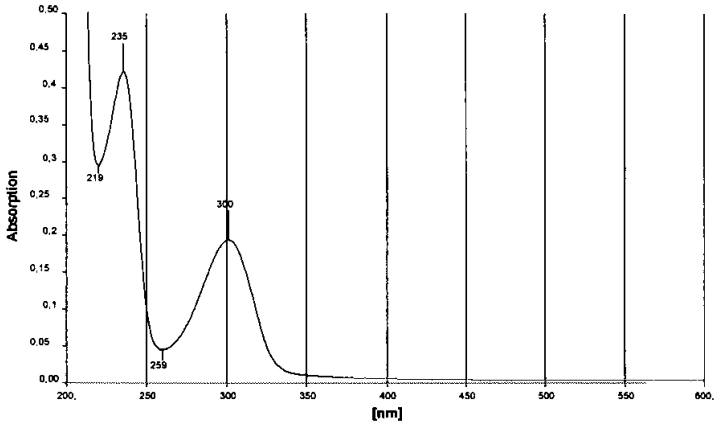

Fig. 3: UV/Vis-spectrum of salicylic acid (200-600 nm) dissolved in the mobile phase.

Table 2

Salicylic acid ($mg \cdot l^{-1}$) in 23 commercial white wines analyzed by HPLC

Variety	Origin	Vintage year	Salicylic acid
Müller-Thurgau	Franken	1997	n.d.
Pinot blanc	Baden	1996	0.01
Pinot blanc	Baden	1996	0.01
Pinot gris	Baden	1996	0.1
Riesling	Baden	1996	0.05
Riesling	Baden	1996	0.01
Riesling	Franken	1997	0.02
Riesling	Mosel-Saar-Ruwer	1997	0.06
Riesling	Pfalz	1997	0.08
Riesling	Rheingau	1996	0.04
Riesling	Rheingau	1996	0.05
Riesling	Rheingau	1996	0.04
Riesling	Rheingau	1997	n.d.
Riesling	Rheingau	1997	0.07
Riesling	Rheingau	1997	0.11
Riesling	Rheingau	1997	0.03
Riesling	Rheingau	1997	n.d.
Riesling	Rheingau	1997	0.03
Riesling	Rheingau	1997	0.10
Riesling	Rheinhessen	1997	0.04
Riesling	Rheinhessen	1997	0.04
Riesling	Rheinhessen	1997	0.04
Silvaner	Franken	1997	0.02

n.d. = not detectable

Wine analyses: SA concentrations of 23 commercial wines are shown in Tab. 2. Compared to the data published in literature, German wines contained very low concentrations of SA. White wines had a mean concentration of 0.05 $mg \cdot l^{-1}$ (0.11 $mg \cdot l^{-1}$ max.). SA was not detected in three samples (detection limit 0.003 $mg \cdot l^{-1}$). The high concentrations reported by Muller *et al.* (1994), ranging from 11.0 to 21.5 $mg \cdot l^{-1}$, were not confirmed. As already assumed by Janssen *et al.* (1997), these high values were probably due to artifacts by coelution during HPLC analysis.

SA has recently received attention as a possible alternative for copper applied against downy mildew in organic viticulture (Wöhrle 1997; Kornitzer 1998) based on its potency to induce systemic acquired resistance "SAR" (Renault *et al.* 1996; Busam *et al.* 1997). Treatments of grapes with or without SA had no influence on the SA content of the resulting must or wine (Tab. 3).

In white wines the maximum SA concentration was 0.04 $mg \cdot l^{-1}$ with a mean of 0.01 $mg \cdot l^{-1}$. Only some red wines had higher natural SA concentrations (up to 0.43 $mg \cdot l^{-1}$, 0.16 $mg \cdot l^{-1}$ mean), but none of these wines originated from experimental plots treated with SA. At the concentrations used in our experiments, no risk of hypersensitivity due to the consumption of wines from SA-treated grapes is possible, as has been shown by Haeberle (1987) with humans at high intake rates of food containing SA.

Because of its antiinflammatory and vasorelaxing effects, SA has partially been made responsible for the health effects of moderate wine consumption (Muller and Fugelsang 1994). Meanwhile it is well known, that the antiinflammatoric and antithrombotic effects in particular can be mainly explained by the acetylation of SA (aspirin®). This esterification leads to acetylation of the cyclooxygenases (COX-1 and COX-2), a subsequent blockade of the synthesis of inflammation mediators, a selective inhibition of thromboxane formation in lower doses, and a relative increase of the production of prostacyclin (PGI-2) with antiadhesive and antiplatelet effects. Yet these effects can not be observed with SA alone (Kalgutkar *et al.* 1998). Moreover, in agreement with Venema *et al.* (1996), we believe that the genuine SA content in wines is generally too low to produce measurable effects *in vivo*. This should always be kept in mind when discussing possible health benefits of SA as well as the fact that SA caused none of the effects reported for acetylsalicylic acid (Kalgutkar *et al.* 1998).

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Table 3

Salicylic acid in must and wine of grapes exposed to various phytosanitary treatments in the field

Treatments (substances)	Variety	Vintage year	Origin	Sample	Salicylic acid ($mg \cdot l^{-1}$)
Organic	Riesling	1998	Rheingau	must	n.d.
Integrated	Riesling	1998	Rheingau	must	n.d.
Salicylic acid	Riesling	1998	Rheingau	must	n.d.
Organic	Riesling	1998	Rheingau	wine	0.01
Integrated	Riesling	1998	Rheingau	wine	0.04
Salicylic acid	Riesling	1998	Rheingau	wine	n.d.
Organic	Riesling	1997	Rheingau	must	n.d.
Integrated	Riesling	1997	Rheingau	must	n.d.
Salicylic acid	Riesling	1997	Rheingau	must	n.d.
Organic	Riesling	1997	Rheingau	wine	0.01
Integrated	Riesling	1997	Rheingau	wine	n.d.
Salicylic acid	Riesling	1997	Rheingau	wine	0.01
Organic	Pinot noir	1998	Baden	must	n.d.
Salicylic acid	Pinot noir	1998	Baden	must	n.d.
Organic	Müller-Thurgau	1997	Baden	must	n.d.
Salicylic acid	Müller-Thurgau	1997	Baden	must	n.d.
Integrated	Cabernet Sauvignon	1998	Rheingau	wine	0.43
Integrated	Dornfelder	1998	Rheingau	wine	0.39
Integrated	Dunkelfelder	1998	Rheingau	wine	0.15
Integrated	Pinot noir précoce	1998	Rheingau	wine	0.31
Integrated	Pinot noir	1997	Rheingau	wine	n.d.
Integrated	Pinot noir	1997	Rheingau	wine	n.d.
Integrated	Pinot noir	1997	Rheingau	wine	n.d.
Integrated	Pinot noir	1997	Rheingau	wine	n.d.

n.d. = not detectable

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